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Interactions between carbohydrate and lipid metabolism in metabolic disorders

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**Acute inhibition of glucose-6-phosphate
translocator activity leads to increased
de novo lipogenesis and development
of hepatic steatosis without affecting
VLDL production in rats**

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Abstract

Glucose-6-phosphatase (G6Pase) is a key enzyme in hepatic glucose metabolism. Altered G6Pase activity in Glycogen Storage Disease and diabetic states is associated with disturbances in lipid metabolism. We studied the effects of acute inhibition of G6Pase activity on hepatic lipid metabolism in unanaesthetized rats. Rats were infused with an inhibitor of the glucose-6-phosphate (G6P) translocator (S4048, 30 mg/kg/h) for 8 hours. Simultaneously, [1-¹³C]acetate was administered for determination of *de novo* lipogenesis and fractional cholesterol synthesis rates by mass isotopomer distribution analysis. In a separate group of rats, Triton WR 1339 was injected for determination of hepatic VLDL-triglyceride production. S4048 infusion significantly decreased plasma glucose (-11 %) and insulin (-48 %) levels and increased hepatic G6P (+201 %) and glycogen (+182 %) contents. Hepatic triglyceride contents increased from 5.8 ± 1.4 $\mu\text{mol/g}$ liver in controls to 20.6 ± 5.5 $\mu\text{mol/g}$ liver in S4048-treated animals. *De novo* lipogenesis was increased more than 10-fold in S4048-treated rats, without changes in cholesterol synthesis rates. Hepatic mRNA levels of acetyl CoA carboxylase and fatty acid synthase were markedly induced. Plasma triglyceride levels increased four-fold but no differences in plasma cholesterol levels were seen. Surprisingly, hepatic VLDL-triglyceride secretion was not increased in S4048-treated rats. These studies demonstrate that inhibition of the G6Pase system leads to acute stimulation of fat synthesis and development of hepatic steatosis without affecting hepatic cholesterol synthesis and VLDL secretion. Results emphasize the strong interactions that exist between hepatic carbohydrate and fat metabolism.

Introduction

Phosphorylation and dephosphorylation of glucose by glucokinase and glucose-6-phosphatase (G6Pase), respectively, are key steps in hepatic glucose uptake and release. The balance between the activities of these enzymes represents an important site for control of hepatic glucose production.^{1,2} G6Pase is located in the endoplasmic reticulum (ER) of liver, kidney and, as recently shown, intestinal cells.³ The glucose-6-phosphate (G6P) metabolizing machinery consists of a putative translocator^{4,5}, responsible for transporting G6P from the cytosol into the ER lumen, and a catalytic subunit that converts G6P to glucose and inorganic phosphate.⁶ The catalytic subunit is localized to the inner ER membrane. Interestingly, there are several indications to suggest that this site of regulation of glucose metabolism is linked to that of hepatic lipid metabolism. G6Pase activity is increased in patients and animal models of Diabetes Mellitus (DM)^{2,7,8}, probably contributing to increased hepatic glucose production in these conditions. Diabetes in general is associated with hyperlipidemia, which has been found to be mainly due to overproduction of very low density lipoprotein-triglycerides (VLDL-triglycerides) in DM type 2 (DM2).⁹⁻¹¹ Deficiency of G6Pase activity, the metabolic basis of Glycogen Storage Disease type I (GSD-1), also leads to abnormalities in lipid metabolism, characterized by severe hypertriglyceridemia and hypercholesterolemia.¹²⁻¹⁵ GSD is caused by mutations in the genes encoding either the putative translocator (type non-1a)^{4,5} or the catalytic subunit (type 1a)^{6,16,17} of the G6Pase system. Overexpression of hepatic glucokinase also leads to hyperlipidemia in fed rats.¹⁸ Brown *et al.* showed that the phosphorylation process is important for regulation of assembly and secretion of triglyceride-containing very low density lipoproteins by hepatocytes.¹⁹ Little is known about the mechanisms underlying the apparent paradox that hyperlipidemia develops in conditions associated with high as well as low G6Pase activity, *i.e.*, diabetes and GSD.

A class of chlorogenic acid derivatives has recently been developed, eventually aimed at treatment of hyperglycemic conditions in DM2, that potently and specifically inhibit G6Pase activity by blocking the translocase of the G6Pase complex.²⁰⁻²² Infusion of members of this class of compounds in anaesthetized rats resulted in reduction of blood glucose levels and increased concentrations of intrahepatic G6P and glycogen.^{21,22} Recently, it has been reported that acute inhibition G6Pase activity also increases hepatic triglyceride concentrations.²³ Due to their mode of action, chlorogenic acid derivatives induce a situation resembling GSD-1 and provide excellent tools to unravel the interactions between carbohydrate and lipid metabolism.

In the present study, we acutely inhibited G6Pase activity by infusion of the chlorogenic acid derivative S4048 *in vivo* in conscious, unrestrained non-diabetic rats. We questioned whether acute increases in hepatic G6P concentrations would lead to increased hepatic *de novo* lipogenesis, cholesterogenesis and VLDL-triglyceride secretion.

Methods

Animals

Male Wistar rats (Harlan Laboratories, Zeist, The Netherlands) weighing between 290-350 g (mean \pm SD: 318 ± 25 g) were used to study the in vivo effects of S4048. To allow infusion and blood collection in freely moving, unrestrained animals, rats were equipped with two permanent heart catheters via the right jugular vein as described by Kuipers *et al.*²⁴ After surgery, animals recovered for a period of 7 days in individual cages in a temperature- controlled room (20 °C) with food and water available ad libitum. At 8h before the start of the experiment, cages were cleaned and food was removed.

Experimental procedures

Animals received an i.v. infusion of the G6P translocator inhibitor (S4048, 30 mg/kg/h, infusion rate 3 ml/h) or of the solvent, phosphate-buffered saline (PBS) with dimethylsulfoxide (DMSO), for 8 hours. Rats were allowed to move freely throughout the experiment and the animals did not show signs of stress. The S4048 compound was synthesized by the chemical department of Aventis Pharma (Frankfurt, Germany).²¹ At the infusion rate employed, S4048 is expected to partially inhibit G6P translocase activity.²¹ Simultaneously, all animals received an infusion of [$1\text{-}^{13}\text{C}$]acetate (0.8 mmol/kg/hr). The infusates were freshly prepared before each experiment. S4048 was dissolved in 20 % DMSO in PBS (vol/vol) and PBS was added to reach a final concentration of 6.1 % DMSO (vol/vol). The solution was then immediately adjusted to pH 7.4. Before the infusion, a basal blood sample (~300 μ l) was taken to determine baseline values of the metabolites studied. During the infusion blood samples (~200 μ l) were taken after 30, 60, 120, 240, 360, 420, and 480 minutes. Samples were heparinized and immediately placed on ice and centrifuged at 5000 rpm for 10 minutes at 4 °C. Plasma was stored at -20 °C until analysis. Despite the relatively high infusion rate, hematocrit levels did not fall significantly during the experiment. At the end of the infusion period, animals were anaesthetized with sodium-pentobarbital and a large blood sample was taken by heart puncture. The abdomen was opened and the liver was rapidly exposed, excised and stored in parts at -80 °C for measurement of G6P and glycogen content, lipid analysis and RNA isolation or rapidly frozen in liquid isopentane for histological analysis (see below).

In vivo VLDL-triglyceride production

The effects of S4048 on hepatic VLDL production were studied in a separate experiment. After the surgical procedures and recovery as described above, rats were infused with S4048 or its solvent and, after 3 hours of infusion, received an intravenous Triton WR 1339 injection (Tyloxapol, Sigma Chemical Co., St. Louis, MO) as a (12 % wt/wt) solution dissolved in saline in a dose of 5 ml/kg body weight. Triton WR 1339 blocks lipolysis of lipoproteins and their accumulation in plasma over time allows calculation of hepatic VLDL-triglyceride production rates.²⁵ To exclude any interference of the solvent containing DMSO on VLDL-triglyceride secretion, a separate group of rats received a

saline infusion. After Triton WR 1339 injection, blood samples were taken after 30, 60, 120 and 180 minutes for measurement of triglyceride concentrations. VLDL production rates were calculated from the slope of the linear triglyceride accumulation curves in time. At $t = 180$ min, a large blood sample was taken for isolation of VLDL/LDL particles and animals were sacrificed. Lipoproteins were isolated according to Pietzsch *et al.*²⁶ using a solution of 15.3 % NaCl and 35.4 % KBr in saline with a density < 1.019 g/ml. Plasma (0.5 ml) was overlaid with 0.6 ml of the NaCl-KBr solution, centrifuged for 100 minutes at 120.000 rpm and 4 °C in a Beckman Optima TLX Ultracentrifuge (Beckman Instruments, Palo Alto, CA) and the VLDL-fraction was collected by tube slicing and frozen until analysis. VLDL particle size was calculated as described by Beil *et al.*²⁷

Analytical procedures

Hepatic lipids were extracted using the Bligh and Dyer method.²⁸ Assay kits for determination of plasma and hepatic triglyceride and cholesterol concentrations were obtained from Hoffmann-La Roche Ltd (Basel, Switzerland) and for determination of plasma and hepatic phospholipid and plasma free fatty acid concentrations from Waco Chemical (Marburg, Germany). Plasma β -hydroxybutyrate was measured using a commercially available kit from Sigma Diagnostics (St Louis, MO). Total protein content of tissue homogenates was determined according to Lowry *et al.*²⁹ Plasma insulin was determined by a radio immunoassay (RIA) RI-13K (Linco Research, Inc., St. Charles, MO). Plasma glucose concentration was determined enzymatically by use of the Beckmann glucose analyzer II (Beckmann Instruments, Palo Alto, CA). Hepatic glycogen was determined after extraction with a 1 mol/l KOH solution by sonication. The extract was incubated for 30 minutes at 90 °C, cooled and brought to pH 4.5 by addition of 3 mol/l acetic acid. Precipitated protein was removed by rapid centrifugation (10.000 rpm, 1min). Glycogen was converted to glucose by treating the samples with amyloglucosidase, followed by assay of glucose at pH 7.4 with ATP, NADP⁺, hexokinase and G6P dehydrogenase.³⁰ Liver samples for the determination of G6P were treated by sonification in a 5 % (w/v) HClO₄ solution. Precipitated protein was removed by rapid centrifugation at 10.000 rpm for 1 min in a cold microcentrifuge and the supernatant was neutralized to pH 7 by addition of small amounts of a mixture of 2 mol/l KOH and 0.3 mol/l MOPS. G6P was determined fluorimetrically with NADP⁺ and G6P dehydrogenase.³¹ Activities of liver enzymes, *i.e.*, alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) were determined by routine chemical chemistry procedures.

Liver histology

To visualize fat deposition in the liver staining with Oil-red-O was performed on 4 μ m frozen liver slices and counterstaining with hematoxylin, according to standard procedures.

GC-MS analysis

For gas chromatography/mass spectrometry (GC/MS) analysis, plasma cholesterol was extracted and derivatized as described elsewhere.³² Palmitate from isolated VLDL fractions was trans-methylated according to Lepage *et al.*³³

Cholesterol and fatty acid derivatives were analyzed on a magnetic sector mass spectrometer (70-250S; VG, Manchester, U.K.) using a Chrompack CP-Sil 19 column (Middelburg, the Netherlands) for assessment of isotopomer distribution patterns. For cholesterol samples, the oven temperature increased from 120 to 260 °C at a rate of 20 °C/min, from 260 to 280 °C at 2.5 °C/min and finally from 280 to 300 °C at 20 °C/min. The ions at *m/z* 368 to 371 were measured under selected ion recording. For fatty acid samples the oven temperature increased from 100 to 300 °C at a rate of 12.5 °C/min. The ions of the palmitate derivative were measured at *m/z* 270 to 272 under selected ion recording.

Calculations

The principle of the MIDA technique is described in detail elsewhere.^{32,35} Briefly, MIDA allows to determine the enrichment of the pool of acetyl-CoA precursor units that has entered newly synthesized cholesterol or palmitate molecules during the course of a [¹³C]acetate infusion, by analyzing the isotopomer pattern of the molecules of interest. This isotopomer pattern is compared with a theoretical table generated using binomial expansion and known isotope frequencies of the atomic isotopes. When the enrichment of the acetyl-CoA pool is known it becomes possible to calculate the fraction (*f*) of newly synthesized cholesterol and palmitate molecules in plasma or VLDL and in liver homogenates. For determination of the absolute amount of newly synthesized hepatic palmitate we multiplied *f* by the total amount of hepatic palmitate at the end of the experiment.

Gene expression studies

Liver samples of approximately 30 mg were used for total RNA isolation with the Trizol method (GIBCO, Paisley, United Kingdom) and the SV Total RNA Isolation System (Promega, Madison, WI). Single stranded cDNA was synthesized using materials from Boehringer Mannheim (Mannheim, Germany), according to manufacturer's instructions. PCR was performed in 50 µl preparations using 3 µl cDNA, 0.25 Taq polymerase, 5 µl tenfold buffer, 0.75 µl dNTP-mix (10 mM) (all from Hoffmann-La Roche), 2 µl DMSO, and 1 µl of each primer (25 pmol, GIBCO). The following primer sets were used: for acetyl-CoA carboxylase (*ACC*, GenBank accession number J03808), sense primer was GGG ACT TCA TGA ATT TGC TGA TTC TCA GTT and anti-sense primer was GCT ATT ACC CAT TTC ATT ACC TCA ATC TC³⁴; for fatty acid synthase (*FAS*, GenBank X13415), sense primer was GGC TTT GGC CTG GAA CTG GCC CGG TGG CT and anti-sense primer was TCG AAG GCT ACA CAA GCT CCA AAA GAA TA³⁴; for sterol regulatory element binding protein-1 (*SREBP-1a* and *1c*, GenBank L16995), sense primer was CCT GTG TGT ACT GGT CTT CCT G and anti-sense primer was ACA AGA TGG CCT CCT GTG TAC T; for *SREBP-2* (GenBank U02031), sense primer was CAA TGG

CAC GCT GCA GAC CCT TG and anti-sense primer was ATG GCC TTC CTC AGA ACG CCA G; for 3-hydroxy-3-methylglutaryl-CoA reductase (*HMG-CoA reductase*, GenBank M29249), the sense primer was GAC ACT TAC AAT CTG TAT GAT G and anti-sense primer was CTT GGA GAG GTA AAA CTG CCA; for *HMG-CoA synthase* (GenBank X52625), sense primer was TAC GAT GGT GTA GAT GCT GG and anti-sense primer was AGT TCT TCT GTG CTT TTC ATC CAC; for apolipoprotein B (*apoB*, GenBank M14952), sense primer was GAC ATG GTG AAT GGA ATC ATG and anti-sense primer was TGA AGA CTC CAG ATG AGG AC³⁴; for β -actin (GenBank M12481), sense primer was AAC ACC CCA GCC ATG TAC G and anti-sense primer was ATG TCA CGC ACG ATT TCC C; for microsomal triglyceride transfer protein (*MTP*, GenBank LA7970)), sense primer was ATC TGA TGT GGA CGT TGT GT and anti-sense primer was CCT CTA TCT TGT AGG TAG TG; for carnitine palmitoyltransferase I (*CPT-I*, GenBank L07736), sense primer was GCA TCA TCA CTG GTG TGT TC and anti-sense primer was TCT CCA TGG CGT AGT AGT TG. For each primer set, an increasing number of PCR-cycles with otherwise fixed conditions was performed to determine to optimal number of cycles, which was chosen as the number halfway through the exponential phase. The PCR products were separated on 2.5 % agarose gels. Images were made using a CCD video camera (Image Master VDS system, Pharmacia, Upsalla, Sweden). Intensities of the bands were quantified using the program Image Master 1D Elite 3.0.

Statistical analysis

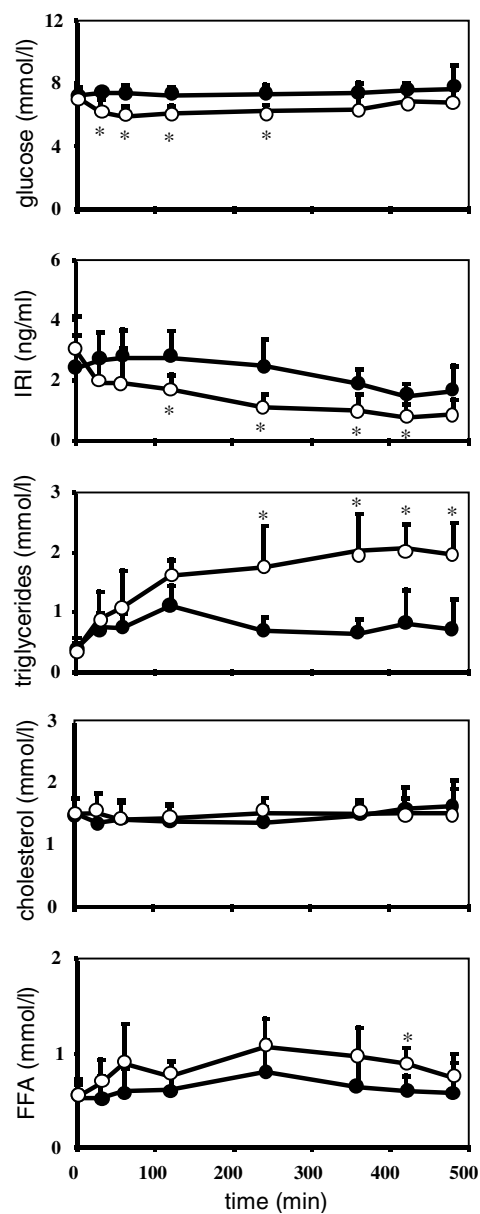
All values reported are mean \pm SD. Significance was determined using the non-parametric Mann Whitney test for unpaired data. Differences were considered significant at $P < 0.05$.

Results

Effects of S4048 on plasma parameters

Figure 1 shows the effects of S4048 infusion on plasma glucose, insulin, cholesterol, triglyceride and free fatty acid concentrations. Infusion of S4048 modestly decreased plasma glucose concentrations ($P < 0.05$), especially during the first two hours of the experiment, with a subsequent significant decrease ($P < 0.05$) in plasma insulin concentrations. Both plasma glucose and insulin concentrations reached values approaching those in control rats at the end of the experiment. Triglyceride concentrations increased significantly during the course of the experiment from 0.4 ± 0.1 to 1.9 ± 0.6 mmol/l in S4048-treated rats. Cholesterol levels did not change during the course of the experiment, while free fatty acid levels displayed a modest increase during S4048 infusion.

Figure 1. Concentrations of plasma glucose, insulin (IRI), triglycerides, cholesterol and free fatty acids (FFA) in rats during an 8 h infusion with S4048 (open symbols) or the solvent (closed symbols) ($n = 6$ in both groups).



Data are means \pm SD. * Indicates significant differences between the two groups.

The ketone body β -hydroxybutyrate concentration was 0.54 ± 0.23 mmol/l in the control and 0.75 ± 0.71 mmol/l in the S4048-treated animals. No effects of DMSO, S4048 or Triton WR1339 administration on ASAT on ALAT activities in plasma were found, indicating absence of direct hepatotoxic actions of these compounds. A modest increase was found in ASAT and ALAT activities in the animals receiving all three compounds simultaneously.

Table 1. Intrahepatic content of G6P, glycogen, triglycerides and cholesterol.

	Control ($\mu\text{mol} / \text{g liver}$)	S4048 ($\mu\text{mol} / \text{g liver}$)
<i>n</i>	8	8
G6P	0.23 ± 0.11	0.70 ± 0.30 *
Glycogen	94.0 ± 64.9	265.4 ± 76.2 *
Triglycerides	5.8 ± 1.4	20.6 ± 5.5 *
Cholesterol	5.3 ± 1.3	5.4 ± 1.6
Cholesteryl esters	0.5 ± 0.2	1.1 ± 0.4 *

Data are means \pm SD. * Significantly different from control rats.

Effect of S4048 on hepatic parameters

Infusion of S4048 clearly affected hepatic carbohydrate and lipid contents (Table 1). Liver weight, expressed as percentage of body weight, was 3.0 ± 0.4 % and 3.3 ± 0.2 % (NS) in the control and S4048 group, respectively. Hepatic G6P and glycogen contents both increased almost threefold after S4048 infusion. Total cholesterol content was not affected, although a higher relative cholesteryl ester content was found. Triglyceride content was markedly increased in the S4048 group, *i.e.*, S4048 induced massive steatosis within the 8 h time frame of the experiment.

Figure 2 shows representative sections of livers from solvent-treated and S4048-treated rats, stained for neutral fat by Oil-red-O. Massive amounts of neutral fat were present in livers of S4048-treated rats, mainly in periportal areas of the hepatic lobuli. In contrast, the relatively small amounts of neutral fat present in the control liver were concentrated around the central veins, *i.e.*, in perivenous areas of the lobuli.

Effect of S4048 on *de novo* lipogenesis and cholesterologenesis

In Table 2 the effects of S4048 on palmitate and cholesterol synthesis rates are summarized. Plasma fractional cholesterol synthesis rates were similar in both groups of rats. Fractional *de novo* lipogenesis in plasma VLDL and liver were increased almost three-fold in the treated animals. When the increased hepatic palmitate content was taken into account, the absolute amount of newly synthesized palmitate was increased > 10-fold

Figure 2. Oil-red-O stained liver sections of a control rat (A) and an S4048-treated rat (B). S4048 treatment results in increased fat deposition in the liver with a preferential localization in the periportal area of the liver lobulus. PP=periportal area, surrounding the portal triad; PV=perivenous area, surrounding the hepatic vein.

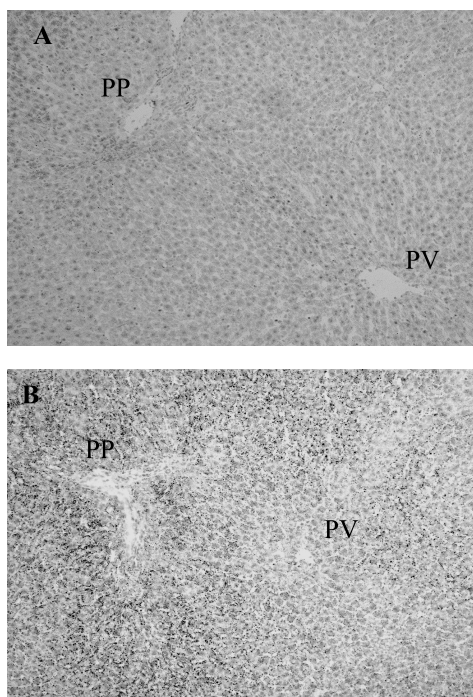


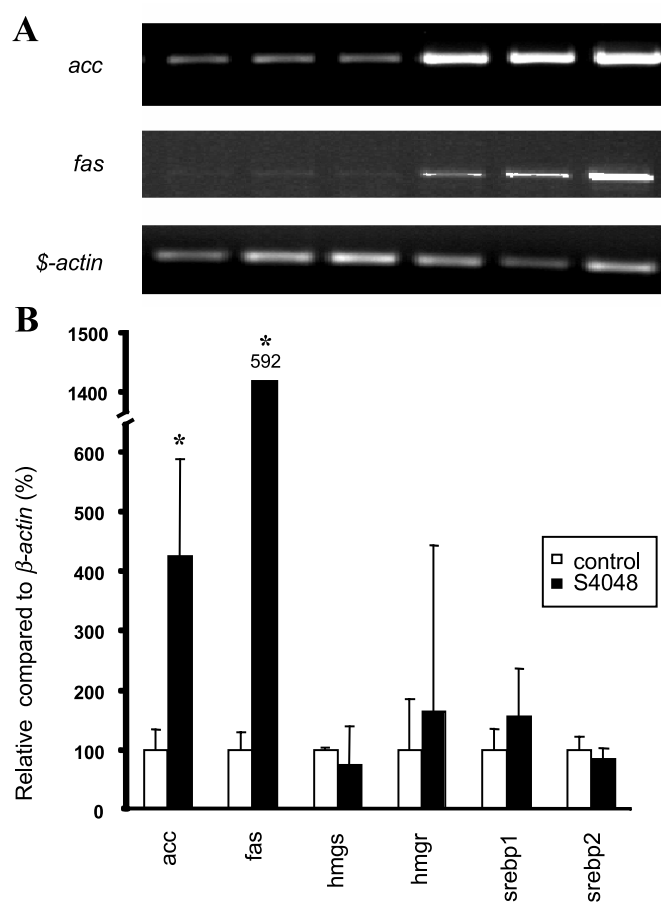
Table 2. Plasma and hepatic fractional cholesterol and palmitate synthesis values, absolute amount of hepatic newly synthesized palmitate and acetyl-CoA pool enrichments in S4048-treated and control rats.

	Control	S4048
Fraction plasma cholesterol (%)	4.0 ± 1.3	5.1 ± 1.2
Fraction of VLDL palmitate (%)	3.0 ± 1.9	7.1 ± 3.8
Fraction of liver palmitate (%)	3.3 ± 1.2	8.7 ± 3.8 *
Newly synthesized hepatic palmitate (nmol/mg	0.2 ± 0.1	2.6 ± 1.2 *
Acetyl-CoA pool enrichments (%)	8.2 ± 1.3	4.4 ± 2.0 *

Data are means ± SD. Samples were obtained from 5 S4048-treated and 6 control rats for all analyses, except for fractional VLDL palmitate synthesis ($n = 3$ for both groups).

* Indicates significantly different from values in control rats.

Figure 3. Image of PCR products of genes upregulated by S4048, *i.e.*, *ACC* and *FAS* in comparison with β -actin (A). The mRNA levels determined by reversed transcriptase PCR ($n = 3$ in both groups) and normalized to β -actin mRNA, expressed as mean percent compared to control values are depicted in B. Levels are shown for *ACC*, *FAS*, *HMG-CoA synthase*, *HMG-CoA reductase* and *SREBP-1* and *2*. * Significantly different from control values.

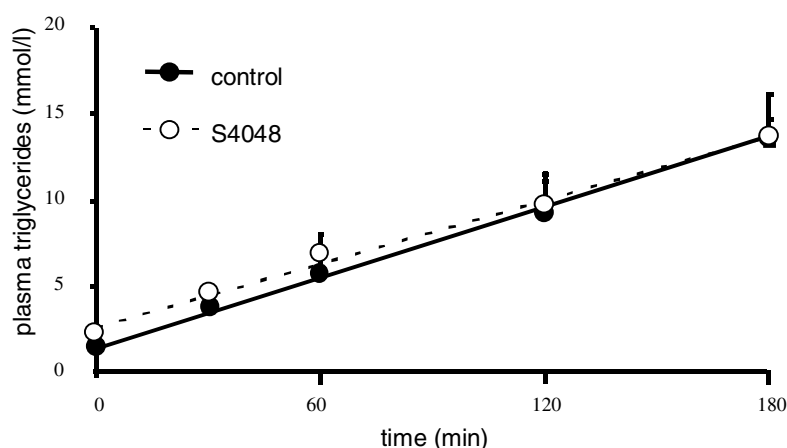


in the S4048-treated group in comparison to the control group. Calculated enrichments of the acetyl-CoA pools showed significantly decreased values in the S4048-treated rats.

S4048 induces expression of lipogenic genes

Figure 3 shows the mRNA levels of *ACC*, *FAS*, *HMGCoA-reductase*, *HMGCoA-synthase* and *SREBP-1* and *2* in control and S4048-treated rats, as determined by a semi-quantitative RT-PCR approach. Intensities of bands were normalized to those of β -actin (Figure 3B). Results clearly show upregulation of mRNA levels of *ACC* (~4-fold) and *FAS*

Figure 4. Determination of very low density lipoprotein-triglyceride production rates. Plasma samples were taken for a period of three hours after Triton WR 1339 injection at $t = 0$ min in three S4048-treated (open symbols) and four control rats (closed symbols) and assayed for triglycerides. Infusion of S4048 or solvent was started three hours before injection of Triton WR 1339. No significant difference was found between the two groups.



(~14-fold) in S4048-treated rats, but no effects on mRNA levels of *HMGCoA-reductase* and *HMGCoA-synthase*. Furthermore, steady state mRNA levels of transcription factors controlling *de novo* lipogenesis and cholesterol synthesis, *i.e.*, *SREBP-1* and 2, were not affected by S4048 infusion. As expected on the basis of unaltered plasma β -hydroxybutyrate concentrations, S4048 did not induce changes in *CPT-1* mRNA levels (data not shown).

Effect of S4048 on VLDL production

In spite of the significant upregulation of hepatic lipogenesis and hepatic lipid content, S4048 infusion did not affect hepatic VLDL-triglyceride production rate (Figure 4): values of 151 ± 33 and 137 ± 14 $\mu\text{mol/kg/h}$ were calculated for control and S4048-treated rats, respectively. Values obtained in the animals infused with DMSO solution alone were similar to those found in rats infused with saline for the same period of time (154 ± 29 $\mu\text{mol/kg/h}$), indicating no effects of the solvent at this dose on VLDL-triglyceride production. The size of the VLDL particles produced by rats treated with the solvent or with S4048 was also similar, with average diameters of 40.7 ± 6.7 nm and 45.9 ± 9.6 nm in control and S4048-treated animals, respectively. As expected, no differences were found in hepatic expression of *apoB* or *MTP* genes (data not shown).

Discussion

Acute inhibition of the G6Pase system by S4048 stimulated hepatic *de novo* lipogenesis more than 10-fold in rats, despite decreased circulating levels of insulin, a well-known stimulator of lipogenesis.³⁷ Increased *de novo* lipogenesis was associated with an increased flux through the acetyl-CoA pool, since lower ¹³C-acetyl-CoA enrichments were calculated for rats treated with S4048. Thus, more substrate for fatty acid synthesis was produced in the S4048-treated rats than in control rats, presumably due to an increased glycolytic flux. Fatty acid synthesis was not only stimulated by increased precursor supply. Concomitantly, hepatic mRNA levels of two key enzymes in *de novo* lipogenesis, *ACC* and *FAS* were markedly induced within the 8 h time frame of the experiment.

Pathways of *de novo* lipogenesis are under transcriptional control of sterol regulatory element binding proteins (SREBP's)^{38,39}, a group of transcription factors (SREBP1a, SREBP1c and SREBP2) that regulate the expression of genes involved in cholesterol, fatty acid and glucose metabolism. SREBP-1 gene knockout mice show a very low basal expression of *ACC* and *FAS* and hardly possess the ability to upregulate *de novo* lipogenesis.³⁸ In contrast, overexpression of the nuclear form of SREBP-1a leads to massive steatosis and increased *de novo* lipogenesis, albeit in absence of hypertriglyceridemia.⁴⁰ In S4048-treated rats *SREBP-1* mRNA expression was not significantly induced, but this obviously does not exclude the possibility of direct SREBP1-mediated activation of gene expression, particularly in view of very recent results by Foretz *et al.*⁴¹ These authors have shown that enhancement of mRNA expression of *ACC* and *FAS* by SREBP-1c in isolated hepatocytes critically depends on the presence of glucose in the medium. Furthermore, it is well established that glucose exerts stimulatory effects of lipogenic gene expression only after being metabolized to either G6P⁴² or xylose-5-phosphate.⁴³ In a recent study it was shown that overexpression of glucokinase in fed rats, leading to increased G6P concentrations, resulted in a marked increase in plasma triglyceride levels.¹⁸ In light of the three-fold increase in G6P concentration in the liver of rats infused with S4048, potentiation of transcriptional activity of SREBP-1 by G6P is highly likely to occur in our model.

Despite increased production of the obligatory precursor, *i.e.*, acetyl-CoA, our stable isotope study showed unaffected cholesterol synthesis rates during infusion of S4048 and unaffected hepatic mRNA levels *HMG-CoA synthase* and *HMG-CoA reductase*. In accordance, overexpression of glucokinase, resulting in an increased hepatic G6P content, did not increase plasma cholesterol levels either.¹⁸ SREBP-2 is a strong regulator of cholesterol synthesis, having the ability to upregulate various genes involved in the cholesterologenic pathway, such as *HMG-CoA synthase* and *HMG-CoA reductase*.^{44,45} *SREBP-2* mRNA levels were not affected in the S4048-treated animals. In combination, these results clearly demonstrate that in spite of common regulatory mechanisms involved, *i.e.* SREBP-modulated activation of gene expression, *de novo* lipogenesis and cholesterologenesis are differentially regulated under conditions associated with increased glycolytic flux in rat liver.

Partial inhibition of G6Pase for an 8 hour period was associated with massive steatosis. Fat accumulation was much more pronounced in hepatocytes located in the zone surrounding the portal vein and hepatic artery (periportal area), than in those surrounding the hepatic vein (perivenous area). This is probably related to the predominant periportal localization of G6Pase.⁴⁶ Increased *de novo* lipogenesis contributed to the development of steatosis, but the quantitative contribution of newly synthesized fatty acids to the steatosis appeared rather limited, *i.e.*, was less than 10 %. Furthermore, VLDL-triglyceride secretion was similar in both groups (see below) and thus did not differentially influence hepatic lipid stores. Increased fat uptake therefore must have contributed, either in the form of VLDL/LDL-triglycerides or as free fatty acids, although plasma free fatty acid concentrations were increased only moderately upon treatment. The latter, however, does not exclude an enhanced free fatty acid flux to the liver. Furthermore, fatty acid oxidation might have been impaired in the S4048-treated rats, although similar mRNA levels of *CPT-I* in livers and unaffected plasma β -hydroxybutyrate concentrations were found. Malonyl-CoA is produced during the course of fatty acid synthesis and is expected to be accumulate in the livers of the S4048-treated rats. Malonyl-CoA is a strong, allosterically acting, inhibitor of fatty acid oxidation⁴⁷, which can thus be achieved without changes in *CPT-I* levels. Furthermore, the increase in hepatic triglyceride content with similar β -hydroxybutyrate concentrations strongly suggests a shift in the balance between fatty acid oxidation and esterification in the liver.

Many factors are known to influence hepatic VLDL-triglyceride production and secretion. *De novo* lipogenesis has been suggested to be of regulatory importance for VLDL production.⁴⁸ Furthermore, many studies have shown that increases in fatty acid delivery to the liver, leading to increased triglyceride synthesis, are accompanied by increases in VLDL secretion.⁴⁹⁻⁵¹ The balance between apolipoprotein B synthesis and degradation is an important factor in controlling hepatic triglyceride secretion and inhibition of protein synthesis has been shown to reduce VLDL-triglyceride secretion.⁵² Additionally, insulin is a well-known acute inhibitor of VLDL secretion⁵³⁻⁵⁵, and insulin resistance is associated with increased VLDL-triglyceride and apoB secretion.⁹⁻¹¹ Furthermore, more recent data has shown that phosphorylation of glucose is also of importance in regulation of assembly and secretion of triglyceride-containing VLDL.¹⁹ In our study, surprisingly, we did not find increased VLDL-triglyceride secretion in the S4048-treated rats. Moreover, neither number nor size of VLDL particles were affected by S4048 infusion. In our model, with induction of *de novo* lipogenesis, strongly increased hepatic triglyceride content in combination with increased hepatic G6P levels and decreased insulin concentrations, increased secretion of VLDL-triglycerides was expected to occur. However, a number of factors have to be taken into account. First of all, it is not known whether apoprotein synthesis was impaired in the S4048-treated rats. Furthermore, if glucose itself is also important in this process, lowered plasma glucose concentrations in the S4048-treated rats might cause an inability to adequately upregulate VLDL secretion. It should be stressed that, in our model, G6P content is increased in the cytoplasm but probably decreased in the endoplasmic reticulum. Compartmentalization of G6P could

potentially play a role in its capability to influence VLDL secretion, but further studies are needed to clarify this phenomenon.

The observation that VLDL-triglyceride secretion was not increased in rats after S4048 treatment indicates that the hyperlipidemia observed after G6Pase inhibition must have originated from decreased triglyceride clearance. Insulin is a well-known stimulator of adipocyte lipoprotein lipase activity. In the S4048 model with low insulin concentration, lipoprotein lipase activity was probably decreased, leading to decreased lipolysis of VLDL-triglycerides. Indeed, studies in GSD patients have shown low LPL activity in GSD patients.⁵⁶

Data in the literature on the relationship between G6Pase activity and lipid metabolism is confusing. Based on our results, we postulate that G6P concentrations in the liver, specifically in certain compartments, play a pivotal role in determining triglyceride concentration in liver and plasma. Altered activation of SREBP-1 and/or changes in the intrahepatic concentration of G6P in itself are most likely of more importance than the plasma concentration of glucose or insulin to explain the apparently conflicting effects of G6Pase activity on hepatic lipid metabolism in diabetes and GSD.

In conclusion, acute inhibition of G6Pase activity in rats leads to increased *de novo* lipogenesis and massive steatosis within a relatively short time frame. Cholesterogenesis was not affected in our study, implying a dissociated regulation of cholesterol and fatty acid synthesis under the conditions employed. Increased *de novo* lipogenesis and hepatic lipid accumulation alone is not sufficient to stimulate VLDL-triglyceride secretion. This study underlines the important function of G6P in control of hepatic lipid metabolism.

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*En toch de beelden van weleer
waarin ik van ons dromen blijf
kleden zelfs de witte cellen
met menselijke vellen*